STUDY PLAN

Pilot Stage Development of a Standardized External Proficiency Testing Procedure for the TZM-bl Neutralizing Antibody Assay

October 3, 2005

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I. Organization and Management

Central Coordination: DAIDS, NIAID, NIH
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Quality Assurance: Duke University Medical Center
Marcella Sarzotti-Kelsoe

Data Acquisition and Statistical Analysis: HVTN-SCHARP
Peter Gilbert
Tom Skillman
Steve Self

Reagent Distribution: Quality Biological, Inc.
Garry Li

II. Introduction

Standardized assessments of the neutralizing antibody response are a critical component of the vaccine discovery process. In the case of HIV-1 vaccines, large numbers of samples will need to be assayed against multiple strains of the virus in different laboratories. A key aspect of this effort will be an ability to achieve and document equivalent assay performance across all laboratories. Recent advances in assay technology have led to major improvements in how HIV-1 neutralizing antibodies are measured. Stable cell lines containing Tat-regulated reporter genes are now available that are susceptible to infection by most strains of the virus and that permit rapid, sensitive and reproducible measurements of neutralization after a single round of infection in a high throughput assay format. Moreover, these assays may be used with molecularly cloned, Env-pseudotyped viruses for greater reagent stability and traceability. A luciferase reporter gene assay performed in TZM-bl (JC53bl-13) cells was recently optimized, and many performance parameters of this assay have been validated. Because this assay is growing in popularity and has been adopted by multiple laboratories, an external proficiency testing procedure is needed that will enable laboratories to become accredited to perform assays for preclinical and clinical HIV/AIDS vaccine trials.

A pilot study has been designed that aims to acquire baseline information on the variability of the TZM-bl assay across laboratories. This pilot study is viewed as the first stage of an iterative process aimed at developing a standard operating procedure (SOP) for inter-laboratory equivalency. It is understood that not all laboratories are using an identical SOP at this time. An evaluation of inter-laboratory variation under these relaxed standardization conditions would inform SOP development and enhance our understanding of assay robustness. Subsequent studies will focus on specific assay parameters that contribute to inter-laboratory variation and investigate ways to minimize this variation. The long-range goal is to implement a standardized program of external proficiency testing that may be utilized by all interested laboratories and will become part of the QA requirements for GCLP laboratories that perform assays for human clinical trials. This overall effort is also expected to yield gold standard reference values for key virologic and serologic reagents that will enhance the quality of proficiency testing.

III. Requirements for Participation

The success of this pilot study will depend on the participation of multiple laboratories. Participation is voluntary. Qualified laboratories must be using a luciferase reporter gene assay in TZM-bl cells according to an established SOP. Prior to enrollment, each participating laboratory will submit a copy of their assay SOP (or detailed protocol) to the designated quality assurance officer: Dr. Marcella Sarzotti-Kelsoe (msarzott@duke.edu). Each participating laboratory will also complete an Assay Checklist (see attached) to be sent to Dr. Sarzotti-Kelsoe at the time that assay results are reported. Participants in this study will comprise an ad-hoc working group that will assist in data interpretation and deciding future studies.

IV. Proficiency Panel

Participants will receive a blinded set of eight serologic reagents shipped on dry ice. The reagents should be thawed immediately prior to use and stored at 4^{o}C without re-freezing until after the proficiency assays have been completed. The samples are to be used without any additional processing (e.g., do not heat-inactivate). Each laboratory will measure the neutralization titer of these serologic reagents against six Env-pseudotyped reference strains of clade B HIV-1 (e.g., QH0692.42, AC10.0.29, PVO.4, WITO4160.33, THRO4156.18, CAAN5342.A2). This proficiency panel of serologic and virologic reagents represents a broad range of antigenic diversity and antibody specificities (Li et al., J. Virol. 79:10108-10125, 2005) that will facilitate inter-laboratory comparisons. Plasmids containing the molecularly cloned Env genes are available from the NIH AIDS Research and Reference Reagent Program (www.aidsreagent.org, catalogue numbers 11018, 11024,

11022, 11033, 11037 and 11038, respectively). Each laboratory will prepare their own stocks of the Env-pseudoviruses using the backbone vector and transfection method of their choice.

V. Other Materials Supplied

Each participating laboratory will receive copies of the Duke Reference Laboratory SOPs for the TZM-bl Neutralization Assay and the Preparation and Titration of Env-Pseudoviruses. Each laboratory also will receive the Duke Reference Laboratory macro (Excel) for data reduction. Use of these SOPs and the macro are optional.

VI. Assay Specifics

Each serologic reagent will be assayed for neutralizing activity against each strain of Env-pseudotyped virus by starting at a 1:20 dilution of antibody (dilution on the virus prior to adding cells) and performing stepwise dilutions such that the final dilution is ≥1:43,740. ID50 and ID80 neutralization titers will be determined from the slope of the neutralization curve. Laboratories are not to share information on their results with other laboratories until the study is completed and the final data have been analyzed.

It is anticipated that not all assays will perform properly and that some assays may need to be repeated. Adequate amounts of certain proficiency reagents may not be available for these repeat assays. Assays that fail to perform properly may be repeated when possible; however, only one set of results should be reported. We ask that results be reported only for assays that performed properly (e.g., based on internal positive controls). Statistical analyses will be adjusted to take into account missing data points from failed assays.

VII. Data Reporting

Results are to be reported as ID50 and ID80 titers directly to Tom Skillman at the HVTN-SCHARP. Results may be submitted electronically using the attached data entry templates.

VIII. Data Analysis

Data will be analyzed by Peter Gilbert at the HVTN-SCHARP. A draft statistical analysis plan is attached. Assay equivalency will be judged based on deviation from expected values (e.g., compared to intra-laboratory variation in the reference laboratory).

IX. Data Interpretations and Future Plans

Information from the assay SOPs and checklists will be used retrospectively to identify specific assay conditions that contributed to inter-laboratory variation. Conditions that result in significant deviations from expected values will be identified and investigated in an unblinded fashion through follow-up experiments as part of a continued assay standardization effort. A common assay SOP will evolve that allows a defined set of flexible conditions to be used that are shown to have no significant effect on assay performance. Likewise, the common SOP will place restrictions on conditions that are essential for uniform assay performance. This common SOP will be validated by additional rounds of proficiency testing to assure that it meets specifications for equivalent assay performance in multiple laboratories. The final stages of this overall plan are to design and validate an SOP for external proficiency testing using the common assay SOP.

TEMPLATE FOR DATA REPORTING

Laboratory: _____

Date of report: _____

Site investigator approving this report: _____

Contact person (if different from site investigator): _____

Data Report Sheet: ID50

	ID50 in TZM-bl cells					
Serologic Reagent	**QH0692.42**	**AC10.0.29**	**PVO.4**	**WITO4160.33**	**THRO4156.18**	**CAAN5342.A2**

Data Report Sheet: ID80

	ID80 in TZM-bl cells					
Serologic Reagent	**QH0692.42**	**AC10.0.29**	**PVO.4**	**WITO4160.33**	**THRO4156.18**	**CAAN5342.A2**

Of the 48 combinations, number of initial assays that failed: _____

When completed, submit this page electronically to:
Tom Skillman (toms@scharp.org)

ASSAY CHECKLIST (to be completed after the performance of assays):

1. **Participating Laboratory**:
2. **Approx. number of TZM-bl assays performed in the past**: **<100 ___; 100 - 1,000 ___; >1,000 ___**
3. **TZM-bl cell passage number**:
4. **Growth medium (include vendor and cat. no.)**:
5. **Method used to split cells (e.g., trypsin/EDTA)**:
6. **Cells used for transfection to generate pseudovirus stocks**:
7. **Transfection method**:
8. **Backbone plasmid**:
9. **TCID50 inoculum**:
10. **Number of TZM-bl cells/well**:
11. **Were cells added directly to virus/sample mixtures (yes/no)?**:

    If "no", how long were cells incubated prior to adding virus/sample mixtures:
12. **Polycation and concentration used**:
13. **Virus + serum incubation time (hrs)**:
14. **Length of infection (hrs)**:
15. **Luciferase reagents (product and manufacturer)**:
16. **Luminometer (model and manufacturer)**:
17. **Method used to calculate ID50 and ID80 (e.g., linear regression, fit curve, etc...)**:

Submit electronically to Marcella Sarzotti-Kelsoe (msarzott@duke.edu)

Participating Laboratories:

Name	Contact Information	E-mail	Sample Set # Confirmation # Shipped/Received
David C. Montefiori, Ph.D.	Department of Surgery SORF Bldg., LaSalle St. Ext. Room 130 Duke University Medical Center Durham, NC 27710 Tel: 919-684-5278	monte@acpub.duke.edu	1, 2, 3 7917 7748 6239 7917 7748 6250 7924 3278 5516 110805 / 110905
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Michael Seaman, Ph.D.	Beth Israel Deaconess Medical Center Division of Viral Pathogenesis 41 Avenue Louis Pasteur RE-118 Boston, MA 02115 Tel: 617- 667-0526	mseaman@bidmc.harvard.edu	5 7912 6547 8510 110805 / 111005
Lynn Morris, Ph.D.	National Institute for Communicable Diseases Private Bag X4 Sandringham 2131 Johannesburg SOUTH AFRICA Tel: +27 11 386 6332	lynnm@nicd.ac.za sarahc@nicd.ac.za *Ship samples to Sarah Cohen at address at left.* Tel: +27 11 386 6341	6 NYC 119250 110905 / 111405
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Victoria R. Polonis, Ph.D.	Walter Reed Army Institute of Research 13 Taft Court, Suite 200 Rockville, MD 20850 Tel: 301-251-8308	vpolonis@hivresearch.org	9 NA 110905 / 111405

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James Binley, Ph.D.	Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, Room 305 San Diego, CA 92121 Tel: 858-909 5142	jbinley@tpims.org	12 7925 7553 6042 110805 / 111505
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Larry Liao/ Barton F. Haynes, MD	Larry Liao PhD, Barton F. Haynes, M.D. Director, Duke Human Vaccine Institute Research Drive 224 CARL Bldg. Box 3258 Duke University Medical Center Durham, NC 27710 Ph: 919-684-5279 Fx: 919-684-5230 hayne002@mc.duke.edu	hayne002@mc.duke.edu hliao@acpub.duke.edu	19 7902 1465 4109 110805 / 110905

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October 3, 2005

Draft Statistical Analysis Plan for a Standardized External Proficiency Testing Procedure for the TZM-bl Neutralizing Antibody Assay

Peter Gilbert
HVTN-SCHARP

Data format for analysis

For each lab the data are arranged in 8 by 6 matrices, with 2 replicates for both ID50 and ID80 responses. The rows are serologic reagents or MAbs (n=8) and the columns are HIV-1 isolates (n=6). The complete replication of both reagents and HIV clones is blinded. The purpose of the replication is to estimate the within-lab component of variation as a reference for calibrating the between-lab component of variation.

For each reagent, two 6-variate vectors lnYID50 and lnYID80 are observed. The following analyses will be done for both lnYID50 and lnYID80; the procedures are described for ln YID50.

Descriptive analyses

1. For each isolate, boxplot of the lnYID50s by lab.

2. For each pair of labs and each isolate, scatterplot of the lnYID50s. Include linear fit line and y=x line. Report Lin's concordance coefficient (combined measure of precision and accuracy that measures deviation from y=x line), and the two components of the concordance coefficient, Pearson's correlation (measures precision) and the component measuring accuracy. A concordance coefficient > 0.95 indicates substantial-to-excellent agreement.

3. For each isolate, Bland-Altman plot of the lnYID50s by lab.

4. Plots of the 8 magnitude-breadth (M-B) lnYID50 curves by lab, including the average M-B curve.

5. For each pair of labs, summarize the discrepancy between the two average M-B curves by one minus the absolute differences in response rates averaged over all the response levels. This summary statistic is the M-Ba (magnitude-breadth agreement).

6. Evaluate descriptively whether the labs give the same rankings of neutralization sensitivities of the 6 isolates, based on mean or median lnYID50's across the 8 reagents.

7. Evaluate descriptively whether the labs give the same rankings of neutralization potencies of the 8 reagents, based on mean or median lnYID50's across the 6 isolates.

8. Evaluate descriptively whether the labs give the same rankings of mean or median area-under-the magnitude-breadth (AUC-M-B) lnYID50 curves.

9. For each lab and each isolate, based on the two replicates, compute the within sum of squares, the between sum of squares, and the total sum of squares. Report the ratio of the between sum of squares and the total sum of squares, which summarizes the intra-lab variation.

10. For each lab, based on AUC-M-B lnYID50 curves and the two replicates, compute the within sum of squares, the between sum of squares, and the total sum of squares. Report the ratio of the between sum of squares and the total sum of squares.

11. For each pair of labs and each isolate, compute the within sum of squares, the between sum of squares, and the total sum of squares. Report the ratio of the between sum of squares and the total sum of squares. The higher the ratio the greater evidence for inter-lab variation.

12. For each pair of labs, based on AUC-M-B lnYID50 curves, compute the within sum of squares, the between sum of squares, and the total sum of squares. Report the ratio of the between sum of squares and the total sum of squares.

13. Based on all labs, compute the coefficient of variation (CV) across labs of various summary statistics.

Inferential analyses

1. For each lab k, test whether it has a distribution of lnYID50 significantly different from the labs-pooled distribution of lnYID50. Formulate a test statistic based on the difference in the 6-vector of average lnYID50s for lab k minus the 6-vector of average lnYID50s pooled over all labs, standardized by the 6 by 6 estimated covariance matrix of this difference (a generalized Wald statistic). Apply a bootstrap procedure to obtain a p-value.

2. Study the distributions of the answers to the questions on lab characteristics to attempt to explain observed differences between labs. Use standard linear-regression variable selection methods to determine the subset of questions that best predict differences between lab-specific lnYID50_j and lab-averaged lnYID50_j for each isolate j, aggregating over the 6 isolates (total of 96 differences). Determine the proportion of variation explained by the set of questions, to evaluate if there are unmeasured factors that explain lab differences (i.e., the set of questions was incomplete). Obtain measures of variable importance to rank the importance of the different questions. Also consider using tree-structured methods (probably random forests).

3. Repeat 1. and 2. using the reference lab (Duke) as the reference instead of the labs-pooled distribution.